Effects of industrial pollution on the reproductive biology of *Squalius laietanus* (Actinopterygii, Cyprinidae) in a Mediterranean stream (NE Iberian Peninsula)

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Abstract

Mediterranean rivers are severely affected by pollutants from industry, agriculture and urban activities. In this study we examined how industrial pollutants, many of them known to act as endocrine disruptors (EDCs), could disturb the reproduction of the Catalan chub (*Squalius laietanus*). The survey was conducted throughout the reproductive period of *S. laietanus* (from March to July 2014) downstream an industrial WWTP located in the River Ripoll (NE Iberian Peninsula). Eighty fish (28 females and 52 males) were caught by electrofishing upstream and 77 fish (33 females and 44 males) downstream a WWTP. For both sexes, the gonadosomatic index (GSI) and gonadal histology were examined and related to water chemical analysis and fish biomarkers. Female fecundity was assessed using the gravimetric method. Fish from the polluted site showed enhanced biomarker responses involved in detoxification. Also, in the polluted site, lower GSI values were attained in both sexes and females displayed lower numbers of vitellogenic oocytes. Gonadal histology showed that all maturation stages of testicles and ovaries were present at the two study sites but fish males from the polluted site had smaller diameter seminiferous tubules. Water chemical analysis confirmed greater presence of EDCs in the river downstream the industrial WWTP. The chemicals benzotriazole and benzothiazole could be partially responsible for the observed alterations in the reproductive biology of *S. laietanus*.

Keywords Endocrine disruption; Benzotriazole; Benzothiazole; Gonadal histology; Female fecundity; Desynchronised spawning.

Introduction

Conservation of freshwater fish has reached a critical point worldwide. In Europe, about 37% of freshwater fish species are included under some threat category of extinction (Freyhof and Brooks 2011). The situation is even worse in the Iberian Peninsula, where roughly 70% of its fish species are endemic (Doadrio et al. 2011). Mediterranean rivers are considered *hotspots* in terms of biodiversity despite being among the most endangered ecosystems worldwide (Cuttelod et al. 2008); with a long-history of anthropogenic insults, including pollution, introduction of exotic species and habitat degradation resulting in an alarming decline of fish populations (Clavero et al. 2004). In Catalonia (NE Iberian Peninsula), two out of the 29 species of native fish are regionally extinct and further 22 (75%) are under threat according to the IUCN (2012). Despite the efforts to implementing the European Water Framework Directive (2000/60/EC) (2000) with wastewater treatment practices, the quality of freshwaters remains worrying. Spillages of wastewater that have not been appropriately treated are still occurring and they invariably lead to the deterioration of water quality downstream (Maceda-Veiga et al. 2013). Moreover, as it is the case of streams in other semi-arid regions, Mediterranean-type climate streams are subjected to summer droughts that can worsen the harmful effects of pollution on the aquatic biota (Colin et al. 2016, 2017). This situation is likely to worsen due to the higher water demand for human use because of climate change (Mekonnen and Hoekstra 2016).

There is a strong body of evidence that an increasing number of chemicals, frequently found in rivers of the Iberian Peninsula, act as endocrine disruptors (EDCs) (Gorga et al. 2015; Kuster et al. 2008; Matamoros et al. 2010a; Osorio et al. 2016). Overall, among the EDCs' effects on the reproductive biology of fish are the occurrence of intersex condition (female oocytes in male testicular tissue), altered oogenesis in females, decreases in fecundity and population recruitment failure (Jobling et al. 1998; Kidd et al. 2014). Some EDCs can interact, even at very low environmental doses (ng/L), with the genesis of fish steroid and the processes

of sexual maturation and differentiation (Vos et al. 2000). Fish reproductive output may be altered by the presence of EDCs in the environment (Nash et al. 2004) and this, in natural environments, may have an strong impact on the ecology and conservation status of native species.

EDCs are widespread in freshwater environments and both laboratory and field based studies have shown reproductive alterations in fish at environmentally relevant exposures. However, it is unclear how these effects may affect fish populations in the wild (Mintram et al. 2018). The main inconvenient of most laboratory studies is that they do not fully reproduce natural conditions and/or do not consider the potential synergy of mixtures of contaminants. Indeed, mixed chemical exposure outcomes can differ significantly from those for single classes of EDCs (Mintram et al. 2018). Moreover, laboratory experiments may not account adequately for vital ecological processes and environmental variations (Galic et al. 2010). The assessment of reproductive disturbances, including histological aspects, is well documented for chemicals with well-known endocrine disruption abilities but the effects may vary depending on concentration, species and sex (Dietrich and Krieger 2009; Sumpter and Johnson 2005). There is a lack of knowledge on how pollutants discharged into Mediterranean rivers may be capable of modifying the reproductive biology of native fish, where most ecotoxicological studies have focused on exotic fish species such as carp (Fernandes et al. 2002; Solé et al. 2003; Lavado et al. 2004). The main aim of this study was to investigate how the presence of EDCs in the water composition of a Mediterranean-type climate stream could alter the reproductive output in a species of native fish. This knowledge is a necessary tool for their conservation. Current approaches for the environmental risk assessment (ERA) of chemicals, including EDCs, lack certainty for protecting wildlife because of differences in species sensitivity to pollutants (Hamilton et al. 2016). Typically, ERA relies on assessments extrapolated from animal laboratory studies at pollutant concentrations not observed in nature (Mintram et al. 2018).

The species selected was the Catalan chub, *Squalius laietanus* Doadrio, Kottelat & de Sostoa 2007, a freshwater fish endemic to the NE Spain and SE France. During the 1990s this species suffered an important decline in the rivers of NE Spain due to pollution, habitat

104 alterations and the introduction of exotic species (Doadrio et al. 2007). Both sexes reach the first
105 sexual maturity when they are 2 years old (females at 10 cm and males at 7 cm long) and the
106 breeding period extends from April to July (Casals 2005). Since the *S. laietanus* population is
107 decreasing, it has been listed as *vulnerable* in both the IUCN (2012) and the Spanish catalogues
108 of threatened species (Royal Decree 139/2011 of 4th February). However, this species has
109 currently a relatively wider distribution (Aparicio et al. 2016), so investigating the effects of
110 pollution on its reproduction can help improve its management and conservation in the future.
111 In this study, the concentration of chemicals potentially acting as EDCs was evaluated through
112 water chemical analyses, and fish stress responses were complemented using biomarkers. The
113 gonadal cycle and the gonadal histology of both sexes, as well as female fecundity, were
114 examined upstream and downstream from the textile dye WWTP, throughout the species
115 breeding period. The results of this study help to clarify the role of pollutants on the freshwater
116 fish decline in semi-arid regions.
117

118 # Methods

119

120 ## Study area

121

122 This study was conducted in the mid stretches of the River Ripoll, a 39.5 Km tributary of the
123 River Besòs basin located in the NE of the Iberian Peninsula. It is a river of Mediterranean-type
124 climate with alternating periods of droughts and flooding. The polluted site was 1.6 Km
125 downstream from the discharge of a WWTP from a textile industry near Castellar del Vallès
126 (41°34′17.88″N, 2°06′01.40″E). The control site, named Les Arenes (41º38′45.05″N,
127 2°03′24.07″E), was 2.7 Km upstream from the WWTP of the textile dye plant (Fig. 1). In the
128 polluted site (thereafter, *downstream WWTP*) the river width during the sampling period was 3-
129 5 m and the average water depth was 20-40 cm. In the control site (thereafter, *upstream WWTP*)

130 the river width was 4-6 m and the average water depth was 20-30 cm. A small dam located
131 between the two sampling sites prevented the passage of fish from one point to another.
132
133 **Site characterisation by water chemical analysis**
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135 Water chemical analysis of this study was carried out following the protocol optimized for
136 freshwater Mediterranean watercourses by Matamoros and Bayona (2006). Water samples were
137 collected at the two sites in March (spring sampling) and July (summer sampling) using pre-
138 cleaned 1 L amber glass bottles. Four water samples were, therefore, collected for chemical
139 analysis. Samples were kept at 4°C, filtered within the next 24 h and processed as previously
140 reported (Matamoros and Bayona 2006). A sample volume of 500 mL was percolated through a
141 previously activated polymeric solid-phase extraction cartridge (200 mg Strata-X cartridge).
142 Elution was performed with 10 mL of hexane/ethyl acetate (1:1). The eluted extract was
143 evaporated to ca. 100 µL under a gentle nitrogen stream, and 186 ng of triphenylamine was
144 added as an internal standard. Finally, the extract was reconstituted to 300 µL with ethyl acetate.
145 Methylation of the acidic carboxyl group was performed in a hot gas chromatography (GC)
146 injector (290°C) by adding 10 µL of TMSH solution (0.25 mol/L in methanol) to a 50 µL
147 sample before injection. Derivatized samples were analysed in a Bruker 450-GC gas
148 chromatograph coupled to a Bruker 320-MS triple quadrupole mass spectrometer (Bruker
149 Daltonics Inc., Billerica, MA, USA) in the electron impact mode (70 eV ionization energy)
150 fitted with a chromatographic column TRB5-MS coated with 5% diphenyl 95%
151 dimethylpolysiloxane (20 m x 0.18 mm i.d., 0.18 µm film thickness) from Teknokroma (Sant
152 Cugat del Vallès, Spain). A 5 µL volume of sample was injected in the PTV mode.
153 Chromatographic conditions, data processing and validation of the methodology have been
154 described elsewhere (Matamoros et al. 2010a; Matamoros and Bayona 2006).
155
156 **Fish sampling**

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158 Fish were sampled by electrofishing using a portable unit which generated up to 200 V and 3 A
159 pulsed D. C throughout the breeding period (March, April, June and July 2014). Samplings took
160 place in the middle of the month. Samplings during April (which took place at the end of the
161 month) were considered representative of the reproductive state of the fish between April and
162 May (Casals 2005). The number of individuals used in each section of this study is specified in
163 Table 1. The fish (except those used in the biomarker analysis) were sacrificed with an overdose
164 of MS-222 and frozen at -20 ° C. All fish were sized (fork length, FL ± 0.1 mm) and weighed
165 (eviscerated mass, W_E ± 0.01 g), and gonads (gonad mass, W_G ± 0.01 g) and liver (liver mass,
166 W_L ± 0.01 g) were removed and weighed. All fishes used in this study were adults. Fish
167 collection was approved by the Regional Government of Catalonia (Ref. AP/007). All
168 applicable international, national, and/or institutional guidelines for the care and use of animals
169 were followed. All procedures were conducted in accordance with the European Directive for
170 animal experimentation (2010/63/EU). One of the co-authors holds a category C FELASA
171 certificate that regulates the use of animals for experimental and other scientific purposes.
172

173 **Site characterisation by the use of fish biomarkers**

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175 A subsample of 36 fish from upstream WWTP and 36 fish from downstream WWTP were
176 transported alive in aerated tanks to the laboratory facilities (Table 1). Fish were sacrificed with
177 an overdose of MS-222. A portion of muscle and the whole liver were frozen in dry ice and
178 liquid nitrogen, respectively. Tissues were stored at -80°C until biochemical determinations.
179 A portion of muscle (≈ 0.2 g) was homogenised in ice-cold 50 mM, and the whole liver
180 was homogenised in 100 mM buffer phosphate (pH 7.4) containing 150 mM KCl, 1 mM
181 dithiothreitol (DTT), 0.1 mM phenanthroline, 0.1 mg/ml trypsin inhibitor and 1 mM
182 ethylenediaminetetraacetic acid (EDTA) at a 1:4 (w:v) ratio using a polytron® blender. The
183 homogenates were centrifuged at 10,000 g x 30' at 4°C and the supernatants obtained (S10)
184 were used for the enzymatic determinations in muscle and liver.

All assays were carried out in triplicate at 25°C, except 7-ethoxyresorufin O-deethylase (EROD) and 7-benzyloxy-4-[trifluoromethyl]-coumarin-O-debenzyloxylase (BFCOD), which were at 30°C, in 96-wellplates using a TECAN Infinite M200 microplate reader (Salzburg, Austria). A detailed description of this multi-biomarker approach has been recently detailed elsewhere (Crespo and Solé 2016). Briefly, EROD and BFCOD activities were measured using 50 µl of undiluted liver homogenate samples (S10) with a reaction mixture containing: 0.2 mM NADPH, 3.3 µM 7-ethoxyresorufin (ER) or 0.02 mM NADPH and 200 µM BFC in 100 mM phosphate buffer (pH 7.4). The reaction was followed over 10 min in 96-wellplates using the fluorescence for hydroxyl metabolites formation at fluorometric conditions as described in the former reference. Carboxylesterase (CbE) activity was measured using 25 µl of the appropriately diluted S10 fraction of the liver using 200 µl of α-naphthyl acetate (αNA; 250 µM final concentration in well) or 1 mM final concentration of ρ-nitrophenyl acetate (ρNPA) as substrates. Glutathione S-transferase (GST) activity was measured in 25 µl of diluted S10 using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The final reaction mixture contained 1 mM CDNB and 1 mM reduced glutathione (GSH). Catalase (CAT) activity was measured using 50 mM H_2O_2. Glutathione reductase (GR) activity was measured using 0.9 mM oxidized glutathione (GSSG) and 0.09 mM nicotinamide adenine dinucleotide phosphate (NADPH) and total glutathione-peroxidase (GPX) activity was measured using 2.5 mM reduced glutathione (GSH), 1 Unit glutathione reductase (GR), 0.625 mM cumene hydroperoxide (CHP) and 0.3 mM NADPH.

In muscle, acetylcholinesterase (AChE) and propionilcholinesterase (PrChE) activities were measured in 4-fold diluted (AChE) or undiluted S10 (PrChE). In each microplate well, 25 µL of sample were mixed with 150 µL of 5,5'-dithio-bis-2-nitrobenzoate (DTNB; 270 µM), and the reaction was initiated by adding 50 µL of the respective thio-substrates, all of them at 1 mM final concentration. Lactate dehydrogenase (LDH) activity was also measured in muscle using 25 µL of diluted S10 and mixed with 150 µL NADH (300 µM) and 50 µL pyruvate (4.5 mM). The total protein contents of the samples for all of the assays were determined following

212 Bradford's method (1976) adapted to microplate, using the Bradford Bio-Rad Protein Assay
213 reagent and bovine serum albumin (BSA) as the standard (0.1-1 mg/mL).

214 Before completing the processing of the samples, the hepatosomatic index (HSI) was
215 calculated according to the formula: $HSI = W_L/W_E \times 100$. This index can provide information
216 related to the fitness of fish, but also on its energy reserves, potential diseases, and exposure to
217 exogenous stresses (Schmitt and Dethloff 2000).

218

219 **Gonadal development and female fecundity**

220

221 The gonadal cycle was described through the gonadosomatic index (GSI) calculated according
222 to the formula: $GSI = W_G/W_E$ x 100. A subsample of 36 fish (21 females and 15 males) from
223 upstream WWTP and a subsample of 37 fish from downstream WWTP (22 females and 15
224 males) were analysed over the breeding period of *S. laietanus* (Table 1). Means and 95% CI of
225 GSI were calculated for sex and site.

226 Oocyte development and female fecundity were examined by the gravimetric method
227 (Bagenal 1978) using the same females that were used for the calculation of the GSI. A portion
228 of one ovary from each female was weighted and transferred into Gilson's fluid and, once the
229 gonads had been separated, oocytes were poured into a column of 18 sieves with a range of
230 mesh-sizes (from 0.15 mm to 1.8 mm) and flushed with running water to facilitate their descent.
231 Based on oocyte diameter frequency distributions, developmental stages corresponding to
232 previtellogenic (immature) and vitellogenic (ripening, or in maturation process, and ripe)
233 oocytes were identified. Fecundity, defined here as the total number of ripening or vitellogenic
234 oocytes in the ovaries prior to spawning (Bagenal 1978), was determined by counting all
235 vitellogenic oocytes in gravid females (*i.e.* in females with ripe oocytes). Mature females could
236 be recognized by the presentation of amber-coloured oocytes (or yolk eggs) in the ovaries.
237 Fecundity (F) was calculated as: $\ln F = a + b \ln FL$ (Bagenal 1978). Since females downstream
238 WWTP were smaller in size, only females in the upstream site that were within a similar range

239 of lengths (from 175 to 215 mm of FL) were taken into account to avoid an underestimation of
240 fecundity in the polluted site. Only mature females prior to spawn (i.e. with amber-coloured
241 oocytes in the ovaries) were considered for the calculation of fecundity. For females
242 downstream WWTP, due to a lack of synchronization of the time of egg laying, females
243 throughout the breeding period were considered for the calculation (N=3 in March, N=3 in
244 April, and N=1 in June). Upstream WWTP females prior to spawn were found in June (N=6).
245

246 **Histological analyses**

247

248 Gonads of 20 females (five per month over the reproductive period of *S. laietanus*) and 15
249 males (five per month except in July, which was not analysed) were used in the histological
250 analyses from upstream WWTP. In the polluted site, gonads of 18 females and 15 males (no
251 males sample in July) were analysed (Table 1). Ovaries and testes were fixed in a 10% Bouin's
252 solution, dehydrated with increasing ethanol, cleared in xylene and finally embedded in paraffin
253 wax (Dietrich and Krieger 2009). Gonad sections of 5 μm were cut on a PFM Rotary 3003
254 microtome (pfm medical, Germany) and stained with conventional Delafield haematoxylin and
255 eosin. Stained gonad sections were examined in an Olympus CH-2 microscope and
256 photographed with a Nikon DS-Ri1 digital camera.

257 Five oocyte stages were defined according to their maturation status: Stage I, primary
258 oocytes; Stage II, large primary oocytes with Balbiani body and pleiomorphic nucleoli-like
259 bodies bordering the nuclear envelope; Stage III, early vitellogenic stage; Stage IV, advanced
260 vitellogenic stage with many variably sized yolk vesicles; and Stage V, mature oocyte with yolk
261 mass occupying the entire oocyte. An additional, non-defined stage that did not fit the former
262 criteria was scored: atretic oocyte, a set of small cells with strong staining and irregular shapes.
263 Frequency and size of each stage of development were calculated following Dietrich and
264 Krieger (2009). For males, four stages of maturation were defined: spermatogonia,
265 spermatocytes, spermatids and spermatozoa. Quantitative methods proposed by Smith (1978)

and Gimeno et al. (1998) were used to determine the percentage of testicular stages and to measure the diameter of seminiferous tubules.

A maturity index for each fish was calculated according to the standardized criteria for ovaries and testes established in Baumann et al. (2013). This is an enhancement to the OECD Histopathology Guidance Document (Johnson et al. 2010). The maturity index gives a fixed value (ranging from zero to one) for each fish according to its maturity stage and it increases as fish sexual maturity progresses.

Statistical analysis

In order to test whether biomarkers and HSI showed differences between sites, a generalized lineal model (GLM) was performed for each of them. The structure of these models was the same for both: a biomarker as the dependent variable and *season* (2 levels: *Spring* and *Summer*) and *site* (2 levels: *upstream* and *downstream*) as factors together with their interaction. The gamma distribution and the identity link function were assumed for all biomarker GLMs. Due to the multiple testing, resulting p-values where adjusted by the method of Benjamini and Hochberg (1995). GSI differences between sites were tested performing a GLM for each sex. Both male and female GLM's used *GSI* as the dependent variable, *month* (4 levels in female GLM: *March*, *April*, *June* and *July*; 3 levels in male GLM: *March*, *April* and *June*) and *site* (2 levels: *upstream* and *downstream*) as factors together with their interaction. The gamma distribution and the identity link function were used.

For the analysis of the variability of the number of oocytes between sites, a GLM was performed per development stage (vitellogenic and non vitellogenic oocytes). Each model used the number of *vitellogenic* and *non vitellogenic* oocytes as the dependent variable. *Month* (4 levels: *March*, *April*, *June* and *July*) and *site* (2 levels: *upstream* and *downstream*) were added as fixed factors together with their interaction. The negative binomial distribution was assumed (Poisson distribution was discarded due to overdispersion). The effect of site on female fecundity was tested by means of a GLM with *number of vitellogenic oocytes* as the dependent

294 variable. The *fork length* was added as a covariate, *site* (2 levels: *upstream* and *downstream*) as
295 factor as well as the interaction between them. The negative binomial distribution and the log
296 link function for count data were assumed (Poisson distribution was discarded due to
297 overdispersion).

298 In order to analyse how the degree of maturity was affected by the WWTP discharges, a
299 generalized lineal mixed model (GLMM) was performed for each sex. Both male and female
300 models had the same structure: *maturity index* was the dependent variable, *month* (4 levels in
301 female GLMM: *March*, *April*, *June* and *July*; 3 levels in male GLMM: *March*, *April* and *June*)
302 and *site* (2 levels: *upstream* and *downstream*) were added as fixed factors together with their
303 interaction. Given that we obtained six maturity index values from each individual (each value
304 from a different gonad section) *specimen* was added to the model as a random factor.

305 The effect of each oocyte development stage on oocyte size was tested performing a
306 GLMM. Each model used cellular size of a stage as the dependent variable. *Month* and *site* (2
307 levels: *upstream* and *downstream*) were added as fixed factors together with their interaction.
308 Levels of the *month* factor were not the same for all models given that the development stages
309 were not present during the same months. *Specimen* was also included as a random factor as we
310 used several measures for each female. The gamma distribution was assumed. In order to test
311 whether the diameter of the seminiferous tubules differed between sites, a GLMM was
312 performed. *Tubule diameter* was the dependent variable and *month* (3 levels: *March*, *April* and
313 *June*) and *site* (2 levels: *upstream* and *downstream*) were the fixed factors together with their
314 interaction. Tubule diameter was measured from six different gonad sections for each specimen,
315 thus *specimen* was added as a random factor. The gamma distribution and the identity link
316 function were assumed.

317 All analyses were conducted in R 3.4.3 (R Core Team 2017). GLMs assuming a gamma
318 distribution were performed with glm() function (package *stats*: R Core Team) and the negative
319 binomial GLM (female fecundity model) with glm.nb()(package *MASS*: Venables and Ripley
320 2002). GLMM was performed with glmer() (package *lme4*: Bates et al. 2015). Non-significant

interactions were removed from final models. Homogeneity and normality of residuals were visually checked for all models.

Results

Site characterisation by water chemical analysis and the use of fish biomarkers

The physico-chemical water parameters at the two sites at the spring and summer samplings are detailed in Table 2. These water quality indicators suggested poorer water conditions downstream WWTP, especially in summer. The chemical analysis of selected contaminants of emerging concern (CEC), including some EDCs, is presented in Table 3. Among the CEC detected, only benzotriazole, benzothiazole and their metabolites were found at high levels in water, particularly at the polluted site at both sampling times.

Results concerning liver and muscle biomarkers are summarized in Table 4. Results in HSI showed a significant effect of the *site* factor ($\chi_1^2 = 25.21,\ \mathrm{p} < 0.001$), being the highest values downstream WWTP. For liver biomarkers, significant effects of the *season* factor for CAT ($\chi_1^2 = 14.29, \mathrm{p} < 0.001$), GR ($\chi_1^2 = 32.71, \mathrm{p} < 0.001$), EROD ($\chi_1^2 = 13.70, \mathrm{p} < 0.001$) and BFCOD ($\chi_1^2 = 46.75, \mathrm{p} < 0.001$) were found, which consisted in higher values in July (summer). The *site* factor showed a significant effect for GR ($\chi_1^2 = 94.08, \mathrm{p} < 0.001$), GPX ($\chi_1^2 = 169.40, \mathrm{p} < 0.001$), CbE-αNA ($\chi_1^2 = 23.42, \mathrm{p} < 0.001$), CbE- ρNPA) ($\chi_1^2 = 16.15, \mathrm{p} < 0.001$), GST ($\chi_1^2 = 17.21, \mathrm{p} < 0.001$), EROD ($\chi_1^2 = 19.82, \mathrm{p} < 0.001$) and BFCOD ($\chi_1^2 = 10.31, \mathrm{p} = 0.002$). All of these biomarkers presented higher levels in the fish collected downstream WWTP. A significant interaction between *season* and *site* factors was only found for BFCOD ($\chi_1^2 = 44.88, \mathrm{p} < 0.001$), which consisted of higher values in summer only downstream WWTP. In muscle, AChE and PrChE biomarkers showed a similar pattern: a significant interaction between *season* and *site* factors (AChE: $\chi_1^2 = 19.06, \mathrm{p} < 0.001$; PrChE: $\chi_1^2 = 7.35, \mathrm{p} = 0.010$) as well as a significant effect of the *season* (AChE: $\chi_1^2 = 116.11, \mathrm{p} <$

348 0.001; PrChE: $\chi_1^2 = 126.59, \mathrm{p} < 0.001$). For LDH a significant effect of the *season* factor was
349 detected ($\chi_1^2 = 57.11, \mathrm{p} < 0.001$) which consisted of a higher value in summer. In summary,
350 biomarkers showed a general tendency to show higher values downstream than upstream
351 WWTP on the one hand, and in summer than in spring on the other.
352
353 **Gonadal development and female fecundity**
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355 Female GSI model showed a significant interaction between *month* and *site* factors ($\chi_2^2 =$
356 $12.30, \mathrm{p} = 0.006$) and a significant effect of the *month* factor ($\chi_3^2 = 24.39, \mathrm{p} < 0.001$).
357 Females upstream WWTP showed maximum GSI values in April and June while females
358 downstream did not reach any peak (Table 5). In males, the *month* ($\chi_2^2 = 8.53, \mathrm{p} = 0.014$) and
359 the *site* ($\chi_1^2 = 12.79, \mathrm{p} < 0.001$) factors were significant showing a maximum in April
360 upstream WWTP (Table 5). In summary, both females and males displayed the highest GSI
361 values upstream WWTP.
362 Size-frequency distributions of previtellogenic (≤ 0.35 mm) and vitellogenic oocytes in
363 maturation process (> 0.35 mm) during the breeding period of *S. laietanus* at the two sampling
364 sites are shown in Fig. 2. A different pattern in size-frequency distributions of vitellogenic
365 oocytes was observed. Females upstream WWTP showed a maximum number of vitellogenic
366 oocytes in June with a peak of mature amber-coloured oocytes (≥ 0.80 mm). By contrast,
367 females downstream had lower amounts of vitellogenic oocytes (in maturation process)
368 throughout the entire breeding period with no clear reproductive peak, and a small group of
369 mature oocytes in April. The statistical analysis of vitellogenic oocytes showed a significant
370 interaction between *month* and *site* factors ($\chi_3^2 = 18.487, \mathrm{p} < 0.001$) as well as a significant
371 effect of the *month* ($\chi_3^2 = 44.256, \mathrm{p} < 0.001$) and *site* ($\chi_1^2 = 6.484, \mathrm{p} = 0.011$) factors. For
372 previtellogenic oocytes a significant interaction between *month* and *site* factors was found
373 ($\chi_3^2 = 12.167, \mathrm{p} = 0.01$) as was a significant effect of the *month* factor ($\chi_3^2 = 35.685, \mathrm{p} <$
374 0.001). Females upstream WWTP had more vitellogenic oocytes than females downstream.

As the covariate *female length* did not affect in the female fecundity model, it was removed from the model. The final model showed a significant effect of the *site* factor ($\chi_1^2 = 13.93, \mathrm{p} < 0.001$): upstream females had about 2.6 times (CI: 1.5 – 4.6) more vitellogenic oocytes than downstream females.

Histological analyses

Females and males collected at both sites displayed all the defined stages of maturation. In Fig. 3, details of the different stages are presented. The female maturity index model showed a significant interaction between *month* and *site* factors ($\chi_3^2 = 8.609, \mathrm{p} = 0.035$), a significant effect of the *month* factor ($\chi_3^2 = 33.44, \mathrm{p} < 0.001$). Both upstream and downstream females showed a quite similar maturation pattern, but upstream females reached a maturation peak in June that was absent for downstream females (Fig. 4). For males, only the *month* factor was significant in the maturity index model ($\chi_2^2 = 16.98, \mathrm{p} < 0.001$). In summary, upstream and downstream males showed an identical maturation pattern which consisted of higher maturity index values in April and June than in March.

Oocyte size expressed as area units (in μm^2) could only be accurately measured from the Stage II of maturation onwards (Table 6). The *site* factor was significant for stages II ($\chi_1^2 = 9.57, \mathrm{p} = 0.002$) and IV ($\chi_1^2 = 3.89, \mathrm{p} = 0.05$), being cells of both stages larger for females downstream WWTP. Occurrence of atretic did not differ per site or season. In the tubule diameter model, only a significant effect of the *site* factor (χ_1^2 = 6.51, p = 0.011) was found, with males upstream WWTP having a higher tubule diameter (mean = 222.99 μm; CI = 184.09 – 261.88) than males downstream WWTP (mean = 164.80 μm; CI = 117.63 – 211.97).

Discussion

401 Former studies carried out in the same area of the Ripoll River, revealed that the site
402 downstream from the textile dye plant, even after wastewater treatment, was still highly polluted
403 (Colin et al. 2016, 2017; Maceda-Veiga et al. 2013; Blanco et al. 2019). In this study, several
404 CEC, such as pharmaceuticals, sunscreen compounds, fragrances, antiseptics, fire retardants,
405 surfactants, pesticides and plasticizers were detected in water. Among them benzotriazole,
406 benzothiazole and their metabolites were found at higher concentrations in the polluted site at
407 both sampling times. Traditional fish biomarker responses considered in this study confirmed an
408 elevation of detoxification enzyme activities and oxidative stress parameters in fish sampled
409 downstream from the WWTP. A study carried out on two cyprinid fish species from the same
410 locations in summer 2012, which also included some of the biomarkers considered here (EROD
411 and BFCOD), reached similar conclusions (Blanco et al. 2019). According to these authors,
412 males from two species (*S. laietanus* and *Barbus meridionalis*) displayed delayed sexual
413 maturation on the polluted site, and females of *B. meridionalis* expressed gonadal aromatase (an
414 enzyme that converts testosterone to estradiol) induction. Our study confirms that, even 2 years
415 later, the endocrine disruption on fish from this area still persists and affects *S. laietanus* as
416 well. The inclusion of fish liver biomarkers confirmed chemical exposures to lipophilic organic
417 microcontaminants: from dioxin-like chemicals (reflected as EROD activity increase) to drugs
418 of broader nature (as BFCOD activity enhancement). Both parameters, together with
419 conjugation enzymes (GST), carboxylesterase activities (CbE) and antioxidant defences (GPX
420 and GR), were significantly elevated downstream from the WWTP, confirming the exposure to
421 a broad nature of chemicals. Nonetheless, the observed differences in biomarkers response
422 between seasons (spring and summer) could be attributed to changes in water temperature and
423 the reproductive status of fish (Van der Oost et al. 2003).

424 Water characteristics in the polluted site may account for the reproductive disorder
425 observed in fish of this study. Females upstream the WWTP followed the expected reproductive
426 pattern characteristic of *S. laietanus* with a maximum number of vitellogenic oocytes in June
427 (Casals 2005). By contrast, females downstream presented vitellogenic oocytes during the entire
428 spawning season with no clear reproductive peak and a reduced amount of mature oocytes in

April, which indicates an alteration of their gonadal cycle and reproductive output. The lower fecundity rates found for females downstream the WWTP were concomitant to a reduced gonadal development relative to body weight (GSI) and to the absence of a peak for this index throughout the breeding period. Significant reductions in GSI in sewage effluent exposed fish have been reported for several species (Hecker et al. 2002; Jobling et al. 1998; Tetreault et al. 2011; Vajda et al. 2008). The histological analysis of females also confirmed the absence of a downstream peak of reproduction. Females collected at both sites showed the five oocytes stages of maturation defined in this study. However, the maturity index (calculated from the proportion of oocytes in the different stages of maturation) was higher for females upstream the WWTP in June but this was not observed in the females downstream. No major histological alterations in the morphology of female gonads were recorded in the current study and in other sewage effluent exposed fish despite the occurrence reproductive alterations (Hecker et al. 2002; Jobling et al. 2002; Solé et al. 2003).

Similar results were observed for males. Males downstream the WWTP had lower GSI values as described by Kaptaner et al. (2016) for another cyprinid fish species. Males of both sites showed the four stages of maturation defined in the histological analysis. Although for males the maturity index was not significantly different between the two sampling sites, on the other hand males in the polluted site had narrower seminiferous tubules. Reduction in diameter of seminiferous tubules has been related to a decrease in male fecundity due to less space to stock sperm (Gimeno et al. 1998; Smith 1978). The lower value of GSI in males downstream may confirm this assumption. Gimeno et al. (1998) reported that a reduction in the diameter of the seminiferous tubules is related to fish exposed to 17 β-estradiol (E2) in *Cyprinus carpio*. Although a high frequency of intersex among males of fish species in wild populations exposed to wastewater discharges has been described (Jobling et al. 1998; Tetreault et al. 2011), feminization in males has not been observed in this study.

In teleost female fish, the process of oogenesis is mostly E2-dependent and oocyte maturation is regulated by the progestin 17α,20β-dihydroxypregn-4-en-3one (17,20β-P). According to Scott et al. (2010), the last one can be affected by environmental contaminants

such as diazinon and synthetic progestogens. An desynchronised spawning reproductive pattern, such as the one revealed for the ovarian development in females of this study, was also reported in other fish field studies due to chemicals of an oestrogenic nature (Vajda et al. 2008; Woodling et al. 2006). However, for males, the effects of EDCs are unpredictable and variable (Leino et al. 2005). Some of the CEC classified as EDCs affect females but not males of teleost fishes (Dietrich and Krieger 2009). The EDCs detected at higher concentrations in the polluted site of this study were benzotriazole (up to 1.3 μg/L), benzothiazole (up to 0.5 μg/L) and their respective metabolites. Both compounds have been detected in several rivers of the Iberian Peninsula such as the Llobregat, Ter, Ebro and Tordera (Herrero et al. 2013; Matamoros et al. 2010a). Formerly, these EDCs were clearly associated with the activity of the textile dye plant of concern (Matamoros et al. 2010b). The antiestrogenic activity of benzotriazole has not been fully demonstrated in fish under experimental conditions (Harris et al. 2007). A laboratory experiment reported a degeneration of the ovaries and a stimulation of spermatogenesis in fish females at concentrations starting from 5 mg/L, while in males an increase in GSI and alterations in seminiferous tubules was found (Liang et al. 2014). However, this concentration was higher than that found in this study downstream WWTP site. Other laboratory studies have revealed that benzotriazole was weakly oestrogenic to fish (Tangtian et al. 2012) but does not cause serious abnormalities in testes (Dietrich and Krieger 2009). Benzothiazole have effects on the structure of the gills and on the swimming of fish (Evans et al. 2000).

The highly prescribed ibuprofen was detected downstream in the summer sampling (134 ng/L). This nonsteroidal anti-inflamatory drug is responsible for causing endocrine alterations in steroidogenic enzymes in an in vitro study for male fish (Fernandes et al. 2011). Ibuprofen has also been related to the induction of vitellogenin in male fish, a fewer broods per pair and with a delay in hatching of eggs in *Oryzias latipes* (Han et al. 2010). Other compounds classified as EDCs (such as bisphenol A, diazinon, oxybenzone, and methylparaben) were found in the polluted site of this study. However these and other compounds (galaxolide and tonalide) were found at concentrations 10 times lower than those that can cause effects on oocyte production (Bhandari et al. 2015; Coronado et al. 2008; Kim et al. 2014; Maxwell and

485 Dutta 2005; Mihaich et al. 2012; Staples et al. 2011) or on testicular properties (Goodbred et al.
486 2015; Staples et al. 2011). The effects of nitrogen compounds on the reproduction of fish are not
487 clear. In females of *Pimephales promelas*, the ammonium can both reduce the production of
488 eggs (Armstrong et al. 2012) as it may not have any effect (Armstrong et al. 2015). In this same
489 species, although it appears that chronic exposure to nitrates can induce a vitellogenic induction
490 in both sexes, it has not been shown that it has effects on the GSI or that it favors the occurrence
491 of intersex between males (Kellock et al. 2018). Nitrate exposure also has no effect on GSI in
492 salmonids (Good et al. 2017). Other studies suggest that neither nitrites nor nitrates have clear
493 effects on fish reproduction (Bjerregaard et al. 2018).

494 In nature, some chemicals could express synergistic or antagonistic interactions with
495 others, and this must be taken into account in field studies as the resulting effects of the mixed
496 exposures could be either under- or overestimated (Ginebreda et al. 2014). Some of these CECs
497 are lipophilic and could be present at higher concentrations in fish than in their environment
498 (Lefebvre 2016). Therefore, the extrapolation of observations from experiments in the
499 laboratory to real polluted environments can fail. Water pollution is one of the main causes of
500 the decline of freshwater fish around the world, but little is known about how mixtures of
501 compounds in water can interact and aggravate their effects on fish. A malfunction in
502 reproduction and a lower fecundity because of EDCs could partly explain the dramatic decline
503 of native fish populations in many freshwater ecosystems. Greater efforts should be directed to
504 enforce the European Water Framework Directive (2000/60/EC) (2000) to avoiding wastewater
505 spillages harmful to fish.

506

507 Conclusion

508

509 Biomarker responses confirmed that fish sampled downstream from the WWTP were
510 undergoing additional chemical stress. Both females and males had lower GSI values
511 downstream the WWTP. Downstream females showed an desynchronised spawning, a lack of

512 reproductive peak and a lower fecundity value. Males had narrower seminiferous tubules. The
513 chemicals benzotriazole and benzothiazole, in mixture with other compounds, could be
514 responsible for the observed reproductive disorders in this fish species although further studies
515 are needed in order to clarify their role as ECDs in nature.
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522

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768

Table 1

Number of fish used in each section of this study: site characterization by biomarkers (B), gonadal development and female fecundity (G & F) and histological analysis (H). Fork length (FL, mean ± S.D) is shown for each sample

		Total	FL (mm)	B	G & F	H
	March	5	143 ± 12.2	5	4	5
Upstream	April	7	170.3 ± 20.9	0	4	5
♀♀(n=27)	June	9	205.9 ± 22.5	0	9	5
	July	6	151.8 ± 11.2	6	4	5
	March	9	178.3 ± 19.4	9	6	5
Downstream	April	5	164.2 ± 19.5	0	5	5
♀♀(n=36)	June	7	154.7 ± 37.4	0	7	5
	July	15	86.4 ± 30.5	15	4	3
	March	11	157.4 ± 18.1	11	5	5
Upstream	April	5	156 ± 14.5	0	5	5
♂♂ (n=35)	June	5	154.4 ± 19.2	0	5	5
	July	14	145.6 ± 12.7	14	0	0
	March	11	152.7 ± 14.4	11	5	5
Downstream	April	5	152 ± 9.8	0	5	5
♂♂ (n=22)	June	5	116.6 ± 10.7	0	5	5
	July	1	-	1	0	0

776 **Table 2**

777 Physico-chemical water parameters upstream and downstream WWTP where fish, *S. laietanus*
778 were sampled during spring (March) and summer (July) 2014

	March		July	
	Upstream	**Downstream**	**Upstream**	**Downstream**
Flow (L/s)	239.5	241.2	128.4	124.0
Temperature (ºC)	14.2	16.9	24.0	23.0
Oxygen (mg/L)	7.23	6.2	7.1	8.03
Conductivity (µS/cm)	728	3,680	709	4,777
pH	8.1	8.3	8.0	8.3
NH_3 (mg/L)	0.04	0.40	0.04	5.30
NO_2 (mg/L)	0.008	0.90	0.008	5.51
NO_3 (mg/L)	0.126	19.6	0.056	10.6
PO_4 (mg/L)	0.1	1.0	0.1	0.8
SO_4 (mg/L)	15.8	414.1	17.9	464.0
Cl (mg/L)	40.0	987.0	31.9	1,088

779

780

781

782 **Table 3**

783 Occurrence of CEC in river water samples (ng/L) during spring (March) and summer (July) 2014.
784 PPCP pharmaceuticals and personal care products. Limit of detection LOD = 3 ng/L. EDCs for
785 which an effect on fish has been described are marked with an asterisk

	March		July	
Compound name	**Upstream**	**Downstream**	**Upstream**	**Downstream**
Azole derivatives				
(corrosion inhibitors)				
5-methyl benzotriazole	<LOD	388	33	61
Benzothiazole*	<LOD	506	50	61
OH-Benzothiazole	27	143	17	265
Benzotriazole*	<LOD	438	92	1,289
Herbicides				
2,4-D	5	4	< LOD	< LOD
Diazinon*	<LOD	135	< LOD	< LOD
Fragances				
Galaxolide*	8	18	20	18
Methyl dihydrojasmonate	37	160	99	38

Tonalide*	9	27	26	13
Cashmeran	5	8	< LOD	< LOD
Flame retardants				
Tri(2-chloroethyl) phosphate	5	34	9	15
Tributyl phosphate	11	25	19	54
Triphenyl phosphate	<LOD	17	< LOD	< LOD
PPCPs				
Bisphenol A*	10	<LOD	< LOD	29
Carbamazepine	<LOD	12	9	11
Ibuprofen*	10	<LOD	< LOD	134
Acetominophen	N.A.	N.A.	< LOD	104
Caffeine	14	51	59	234
Methylparaben*	<LOD	<LOD	23	104
Oxybenzone*	<LOD	11	< LOD	13

786

787

788 **Table 4**

789 Biomarkers analysed in liver and muscle of *S. laietanus* at the two sampling sites (upstream
790 and downstream WWTP) during spring (March) and summer (July) 2014. Number of replicates
791 (n) and Means ± SE are shown. Activities of EROD: 7- ethoxyresorufin O-deethylase, BFCOD: 7-
792 benzyloxy-4-[trifluoromethyl]-coumarin-O-debenzyloxylase, CbE: carboxylesterase, GST:
793 glutathione S-transferase, CAT: Catalase, GPX: total glutathione-peroxidase, GR: glutathione
794 reductase, AChE: acetylcholinesterase, PrChE: propionilcholinesterase, LDH: lactate
795 dehydrogenase. The hepatosomatic (HSI) index is also indicated. Units of enzymatic activity:
796 1pmol/min/mg prot, 2nmol/min/mg prot, 3µmol/min/mg prot. In order to test statistical
797 differences, a GLM (generalized lineal model) was performed for each variable. For each
798 variable, not significantly different observations are labelled by the same letter. All significant
799 differences are $p \leq 0.05$

	March		July	
	Upstream	**Downstream**	**Upstream**	**Downstream**
	(n = 16)	(n = 20)	(n = 20)	(n = 16)
HSI	1.0 ± 0.1a	1.3 ± 0.1b	0.9 ± 0.1a	1.2 ± 0.1b
Biomarkers in liver				
EROD[1]	1.6 ± 0.2a	3.0 ± 0.3b	2.7 ± 0.3b	4.1 ± 0.4c

BFCOD[1]	1.5 ± 0.2a	2.7 ± 0.3b	1.4 ± 0.2a	10.9 ± 1.9c
CbE-aNA[2]	312.1 ± 11.2a	376.9 ± 12.0b	313.2 ± 10.3a	378.0 ± 12.6b
CbE-pNPA [2]	510.4 ± 41.4a	742.7 ± 51.3b	611.9 ± 43.8a	844.1 ± 57.0b
GST[2]	692.2 ± 35.7a	872.6 ± 38.8b	696.5 ± 32.9a	876.9 ± 41.4b
CAT[3]	1050.9 ± 55.6a	1143.4 ± 54.5a	1309.1 ± 60.0b	1401.6 ± 66.5b
GPX[2]	210.8 ± 9.0a	372.3 ± 12.6b	211.6 ± 8.2a	373.0 ± 13.0b
GR[2]	25.3 ± 1.0a	40.0 ± 1.3b	33.3 ± 1.1c	48.1 ± 1.5d
Biomarkers in muscle				
AChE[2]	45.8 ± 2.9a	46.0 ± 2.6a	74.3 ± 4.1.8b	115.0 ± 7.2c
PrChE[2]	12.7 ± 0.8a	12.5 ± 0.7a	26.1 ± 1.5b	33.7 ± 2.2c
LDH[2]	1119.1 ± 100.1a	1135 ± 39.6a	2320 ± 167.3b	1918.1 ± 154.7b

800

801 **Table 5**

802 Gonadosomatic Index (GSI) values for females and males of *S. laietanus* at the two sampling
803 sites (Upstream and Downstream WWTP). For males, no data from July was analysed. Means
804 and 95% confidence interval (CI) are shown. A GLM (generalized lineal model) was performed
805 for each sex. Not significantly different observations are labelled by the same letter. All
806 significant differences are $p \leq 0.05$

807

	GSI ♀♀				**GSI ♂♂**			
	Upstream		**Downstream**		**Upstream**		**Downstream**	
	Mean	CI 95%	Mean	CI 95%	Mean	CI 95%	Mean	CI 95%
March	4.76a	3.46 - 6.06	3.47a	2.42 - 4.52	4.40a	2.97 - 5.82	2.14a	1.23 - 3.04
April	5.87b	4.27 - 7.46	4.58a	3.02 - 6.13	6.35b	4.51 - 8.18	4.09a	2.49- 5.68
June	6.62b	4.97 - 8.27	5.33a	3.74 - 6.93	4.35a	2.93 - 5.77	2.09a	1.20 - 2.97
July	2.89a	1.87 - 3.91	1.60a	0.94 - 2.26	-	-	-	-

808

809 **Table 6**

810 Oocyte size for the four stages of development and atretic oocyte of *S. laietanus* expressed in
811 μm^2. Means and 95% confidence interval (CI) are shown. In order to test statistical differences,
812 a GLMM (generalized lineal mixed model) was performed for each sex. * Significant
813 differences by site. All significant differences are $p \leq 0.05$

814

	Upstream		**Downstream**	
	Mean	CI 95%	Mean	CI 95%
Stage II	111.36*	85.31 - 137.42	233.23	203.68 - 262.78
Stage III	2380.28	2042.90 - 2717.65	2767.76	2423.73 - 3111.80
Stage IV	791.57*	155.71 - 1427.43	2079.36	1499.42 - 2659.31
Stage V	5729.56	4878.79 - 6580.33	5137.75	3929.48 - 6346.02
Atretic	81.94	74.05 - 89.83	85.52	77.02 - 94.01

815

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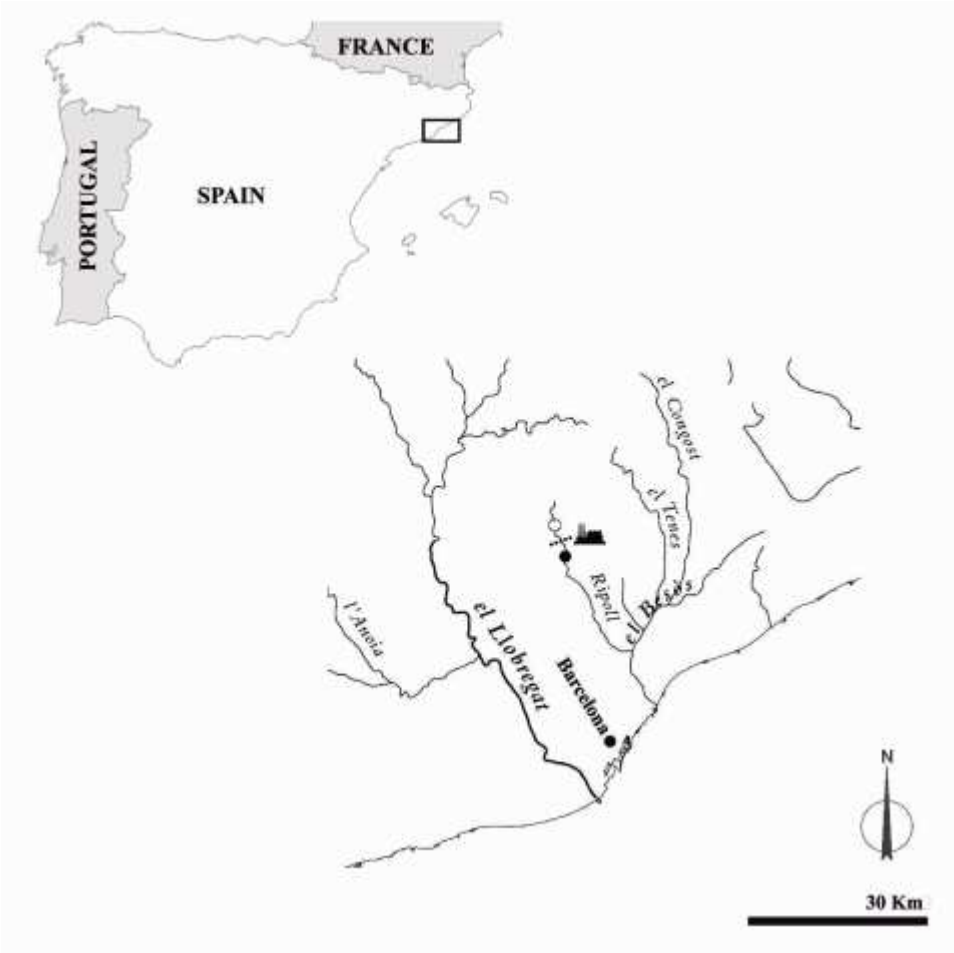

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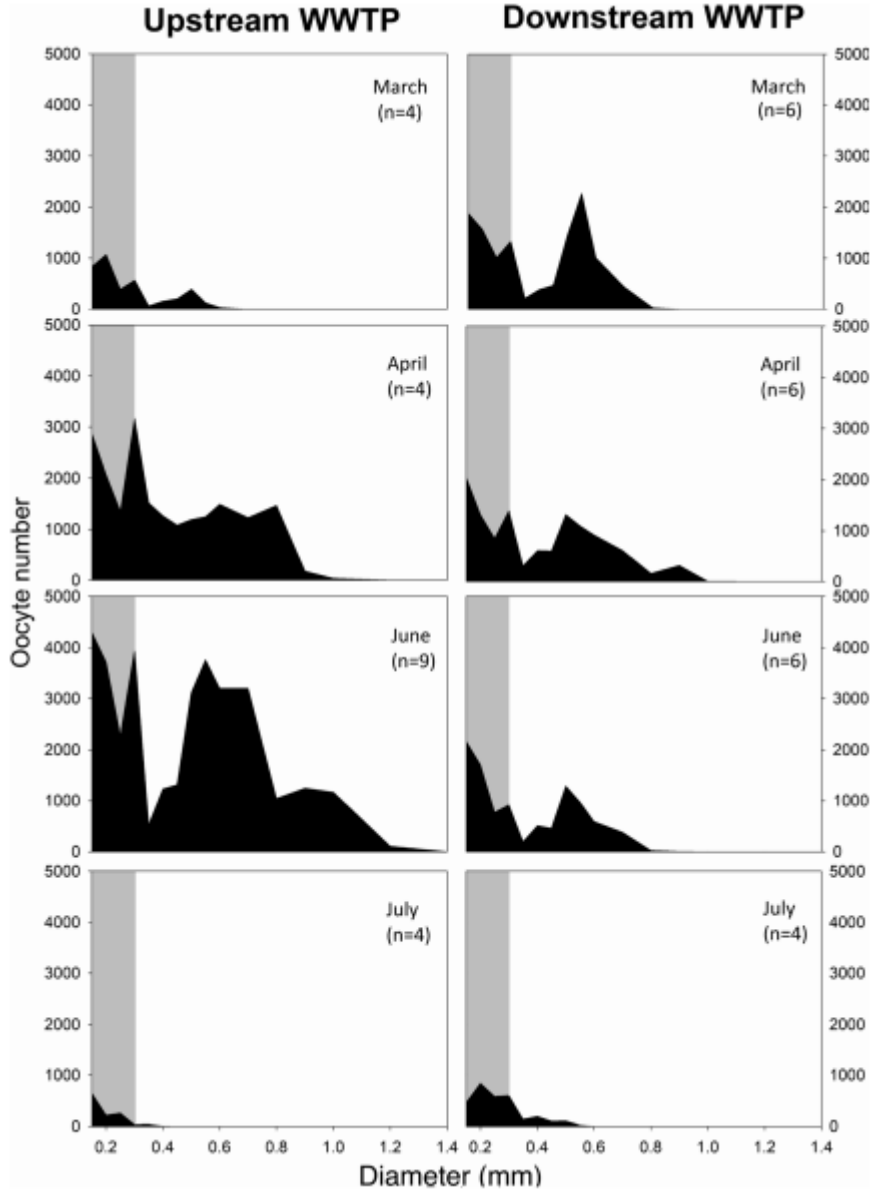
818

819 **Fig. 1** Map of the sampling sites in the Ripoll River (Besòs basin, NE of the Iberian Peninsula).
820 The black point indicates the location of the polluted site (1.6 Km downstream from the WWTP
821 of a textile dye plant) and the white point indicates the location of the control site (2.7 Km
822 upstream from the WWTP). A small dam (dashed line) located just above the textile dye plant
823 (factory symbol) prevented the passage of fish from one site to the other

824



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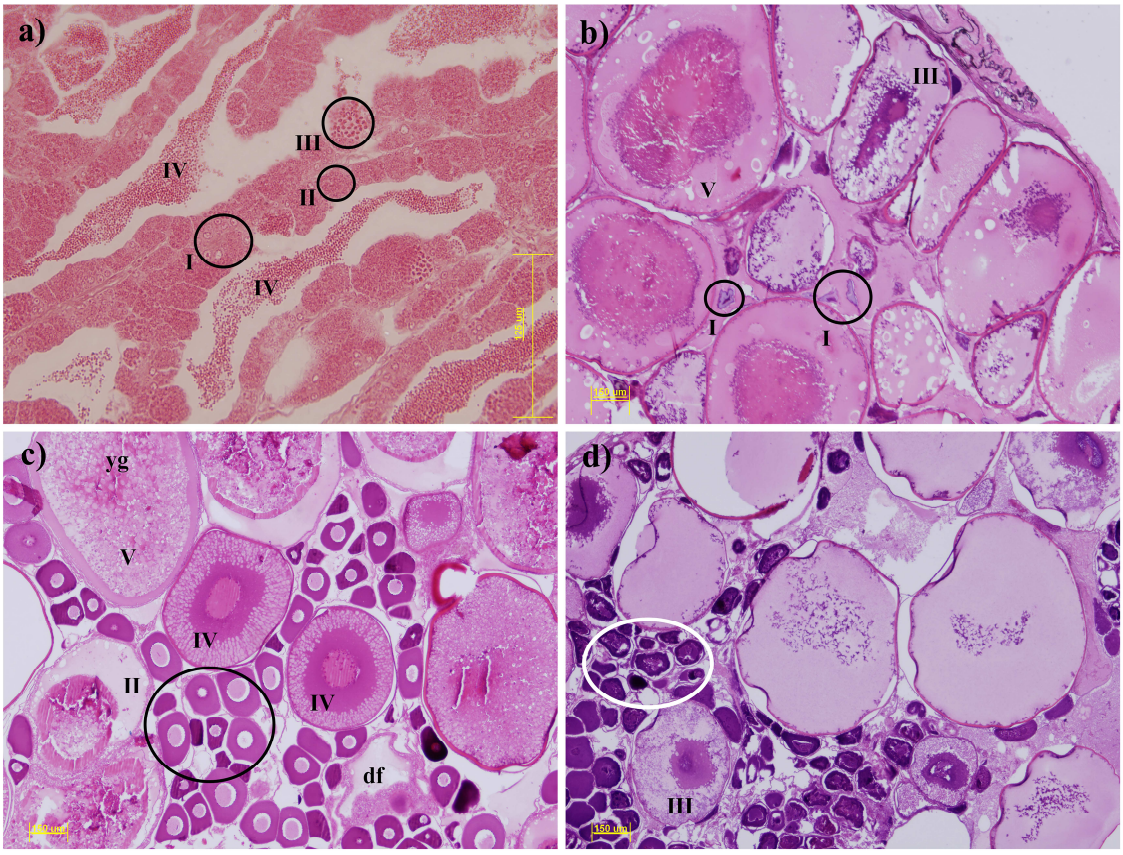
826

827 **Fig. 2** Size distributions of oocytes over the reproductive period of *S. laietanus* in the two
828 studied sites (upstream and downstream from the WWTP) of the Ripoll River. Previtellogenic
829 oocytes ≤ 0.35 mm in diameter (grey area) and vitellogenic oocytes > 0.35 mm in diameter
830 (white area) are shown. Females upstream WWTP showed a peak of ripe oocytes (> 0.8 mm) in
831 June. Females downstream had lower amounts of vitellogenic oocytes (in maturation process)
832 throughout the entire breeding period with no clear reproductive peak, and a small group of
833 mature oocytes in April

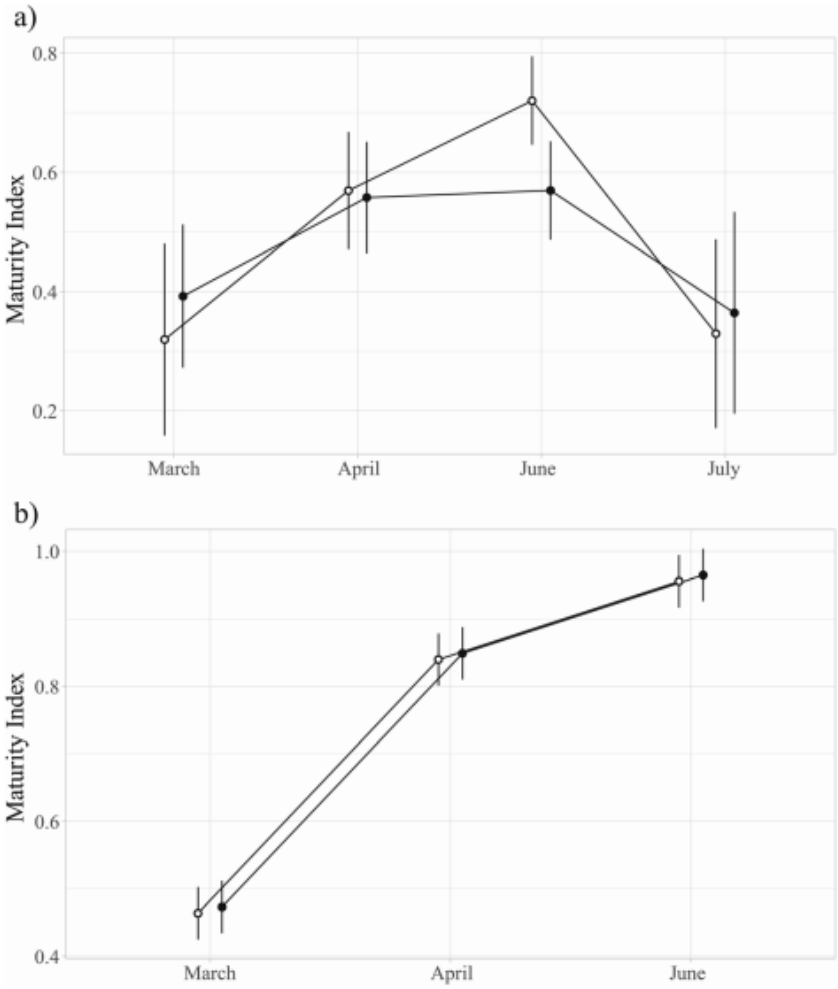
834



835

836

837 **Fig. 3** Photomicrographs of *S. laietanus* gonadal sections stained with haematoxylin and eosin
838 stain, showing ovaries and testes at different developmental phases. (a) Mature testicle, showing
839 spermatozoa (IV) at the centre of the seminiferous tubule and all the stages of development:
840 spermatogonia (I), spermatocytes (II) and spermatids (III) (upstream male). (b) Mature ovary,
841 showing oocytes in phase V (vitellogenic), as well as phase III and phase I (upstream female).
842 (c) Overview of female gonad where phase II, phase IV and phase V (vitellogenic) oocytes are
843 shown (upstream female). (d) Detail of an atretic oocyte (AO) found in some specimens of the
844 study and phase I and phase III oocytes (downstream female). Black and white circles indicate
845 maturation phases with a small size. yg: yolk granule, df: follicles after spawn



846

847 **Fig. 4** (a) Maturity Index for *S. laietanus* females over the reproductive period, shown on a 0 to
848 1 scale. White circles correspond to females upstream WWTP showing a maturation peak in
849 June. Black circles correspond to females downstream WWTP not showing this peak. (b)
850 Maturity Index for *S. laietanus* males over the reproductive period represented on a 0 to 1 scale.
851 White circles correspond to males upstream WWTP. Black circles correspond to males
852 downstream WWTP. Males from upstream and downstream WWTP showed the same
853 maturation peak in June. Average values and 95% confidence intervals (CI) are shown